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(54) Title: ADENOVIRAL VECTORS MODIFIED FOR INCREASED AND PERSISTENT EXPRESSION OF THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR GENE IN HUMAN AIRWAY EPITHELIA

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(57) Abstract: Disclosed are recombinant viral vectors for gene transfer to the epithelium of patients. Preferred embodiments include adenoviral vectors designed for the efficient transfer of the DNA encoding the cystic fibrosis transmembrane conductance regulator (CFTR) to human airways *in vivo*. The present invention provides vectors for the enhanced expression and persistence of a gene such as CFTR, which may allow lower vector doses and/or longer intervals between readministration.

ADENOVIRAL VECTORS MODIFIED FOR INCREASED AND PERSISTENT EXPRESSION OF THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR GENE IN HUMAN AIRWAY EPITHELIA

FIELD OF THE INVENTION

The present invention relates to adenoviral vector constructs for use in gene therapy. More specifically, the present invention relates to vectors useful for tissue-specific expression of transgenes in epithelial tissue.

BACKGROUND OF THE INVENTION

Cystic fibrosis (CF) is a genetic disease resulting from mutations in the chloride channel (Cl^-) encoded by the cystic fibrosis transmembrane conductance regulator (CFTR) gene [Quinton, 1990; FASEB J. 4: 2709 – 2717; Riordan, 1993; Annu. Rev. Physiol. 55: 609 – 630]. The apical membranes of CF airway epithelia lack functional CFTR, resulting in a loss of cAMP-mediated transepithelial Cl^- transport [Welsh, and Smith, 1993; Cell 73: 1251 – 1254]. Gene therapy for CF would require the delivery of a corrected CFTR gene to the airway in sufficient quantity to result in a measurable therapeutic index [see Boucher, 1999; J. Clin. Invest. 103: 441 – 445]. One gene delivery vehicle that shows promise due to its respiratory tropism and relative safety is a recombinant adenovirus in which the viral transforming genes are replaced with CFTR cDNA. Although numerous studies have shown the efficacy of adenoviral-mediated CFTR transfer to animal airway cells [see, Goldman, et al. 1995; Human Gene Ther. 6: 839 – 851; Rosenfeld et al., 1992; Cell 68:143–155; Scaria et al., 1998; J. Virol. 72:7302–7309], the results in human cells have been variable, both *in vitro* and *in vivo* [Crystal et al., (1994); Nature Genetics 8, 42– 51; Grubb et al., 1994; Nature 371: 802 – 806; Zabner et al., 1993; Cell 75: 207– 216]. Potential limiting issues include inefficient viral entry into airway cells and vector immunogenicity [Arcasoy et al., 1997; Am. J. Respir. Cell Mol. Biol. 17: 422 – 435; Goldman and Wilson, 1995; J. Virol. 69: 5951- 5958; Pickles et al., 1996; Hum. Gene Ther. 7: 921 931; Pickles et al., 1998; J.Virol. 72: 6014 – 6023; Walters et al., 1999; J. Biol. Chem. 274:10219- 10226; Yang et al., 1995; J.Virol. 69:2004 – 2015; and Zabner et al., 1997; J. Clin. Invest. 100:1144–1149]. Newer versions of vectors are being developed in which additional viral genes are deleted to reduce cytotoxic host responses [Goldman et al., 1995; Human Gene Ther. 6: 839 – 851; Kochanek et al., 1996; Proc. Natl. Acad. Sci. USA 93:5731-5736; Schiedner et al., 1998; Nature Genetics 18:180 – 183] and ligands with known or novel binding potential

are being added to target the vector to cells of choice [Dmitriev, et al., 1998; J.Virol. 72:9706 – 9713; Romanczuk et al., 1999; Human Gene Therapy, 10(16):2615-26].

Strong viral enhancers and promoters have been used extensively in many applications to achieve high levels of gene expression *in vitro* and *in vivo*. The cytomegalovirus (CMV) enhancer/promoter is frequently used because of its recognized strength and pan-activity [Schmidt et al., 1990; Mol. & Cell. Biol. 10:4406 – 4411]. Its value for providing high level gene expression, however, is often negated by its silencing over time in both immunocompetent and immunodeficient mice [Armentano et al., 1997; J.Virol. 71:2408-2416; Brough et al., 1997; J. Virol. 71:9206-9213, Schmidt et al., supra].

Previous analyses of adenoviral transduction of human airway epithelia have shown that gene delivery is inefficient, most likely due to the paucity of receptors necessary for virus binding and internalization on the apical cell surface [Goldman and Wilson, 1995, J. Virol. 69:5951-5958; Pickles et al., 1998, J.Virol. 72:6014-6023; Walters et al., 1999, J. Biol. Chem. 274:10219-10226; Zabner et al., 1997, J. Clin. Invest. 100:1144–1149]. Strategies to improve adenoviral gene delivery to the lung have made use of novel mechanisms of increased vector attachment and internalization by genetic or chemical modifications to the virus [Dmitriev et al., 1998, J.Virol. 72: 9706–9713; Fasbender et al., 1997, J. Biol. Chem. 272:6479-6489; Kaplan et al., supra; Lee et al., 1999, Hum. Gene Ther. 10: 603 – 613; 38. Romanczuk et al., supra; Wickham et al., 1997, J. Virol. 71:8221–8229]. These types of modifications are predicted to increase the efficiency and specificity of vector delivery by targeting novel or alternate receptors.

SUMMARY OF THE INVENTION

The chronic nature of a lung disease like cystic fibrosis [CF] necessitates further improvements in gene delivery vectors, and that is effective and persistent expression of the therapeutic gene. The effect of regulatory elements such as promoters and transcriptional and translational enhancers thus may be paramount in importance for the duration of gene expression during the lifetime of transduced well-differentiated airway epithelial cells. The frequent turnover of airway epithelial cells will necessitate vector readministration for the treatment of any chronic lung disease. Maximizing gene expression while minimizing cytotoxic vector properties will thus be important in determining the interval between vector readministration. Vectors in which all viral genes have been deleted have been shown to minimize cytotoxicity and increase persistence [Kochanek et al., 1996, PNAS USA 93:5731–5736; Schiedner et al., 1998, Nature Genetics 18:180-183]. These types of modifications, together with the changes within the

transgene cassette described in the present invention, can greatly enhance the efficiency of therapeutic gene expression within human airways. The relative resistance of airway cells to invading foreign agents, the chronic nature of cystic fibrosis, and the relatively rapid turnover of airway epithelial cells will necessitate the implementation of several strategies for efficient therapeutic gene delivery.

Presently, a need exists for increased efficiency of expression of CFTR in targeting human airway cells. Accordingly, the present invention provides methods for increasing the efficiency of expression of CFTR in human airway cells. In particular, methods having increased efficiency of targeted expression of CFTR in human airway cells are provided by incorporating into the transgene cassette a tissue-specific enhancer/promoter for stable and persistent gene expression and *cis* elements for more efficient and sustained mRNA processing.

In the present invention, a regulatory element from a gene with a defined tissue-specificity and a proven pattern of persistence in the lung is demonstrated to better direct long-term CFTR expression in targeted ciliated human airway epithelia cells. The human keratin 18 (K18) gene is expressed in a variety of adult epithelial tissues including the lung [Broers et al., 1989; Differentiation 40:119-128]. Sequences flanking the coding region have been defined for their role in tissue specificity, insulation of gene expression, and copy number dependent expression [Neznanov et al., 1993; Mol. Cell. Biol. 13:2214 – 2223].

Accordingly, in one embodiment, the present invention comprises an adenoviral expression construct comprising, in operable linkage, the K18 promoter/enhancer element, an intron and a stability enhancing element. In preferred embodiments, the adenoviral expression construct additionally comprises a transgene. The transgene may preferably encode human cystic fibrosis transmembrane conductance regulator gene, or an antimicrobial peptide. In other preferred embodiments, the intron comprises a hybrid intron which contains a truncated tripartite leader and splice donor site from adenovirus and a splice acceptor site from a mouse immunoglobulin gene; the stability enhancing element is the α-globin mRNA stability element and additional expression enhancing elements may be included. In preferred embodiments, these expression enhancing elements may be *cis*-acting expression enhancing elements, such as nuclear export elements, splicing enhancers, post-transcriptional regulatory elements, and 5' or 3' untranslated regions [UTR]. The present invention also includes methods for increasing expression of a transgene in epithelial cells in a patient, said method comprising administering to a patient the expression constructs of the present invention.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1. The α-globin mRNA stability element increases CFTR expression. A 110 base pair element from the human α-globin gene was cloned downstream of the CFTR trancription stop site, upstream of a synthetic BGH polyA site (a). 293 cells were infected at an MOI of 5 with Ad2/CFTR-16 or Ad2/CFTR-19. 48 hours post-infection, cell lysates were isolated and CFTR protein was recovered and analyzed on a 6% SDS polyacrylamide gel (b). Arrow shows the expected migration of CFTR, based on protein molecular weight markers. The 293 lane depicts background protein levels in uninfected cells.

FIGURE 2. Persistence of CFTR expression *in vivo*. BALB/c mice were instilled intranasally with $1x10^9$ infectious units Ad2/CFTR-16 or Ad2/CFTR-19. CFTR expression in mouse lungs was analyzed by RT/PCR at 3, 21, and 47 days post-instillation.

FIGURE 3. Expression of βgal from the K18 enhancer/promoter *in vivo*.
Lungs from BALB/c mice instilled with Ad/K18/βgal were analyzed 3 days later for ß-gal expression using x-gal staining of gross (a, b) and sectioned (c) lung specimens.

FIGURE 4. Expression of CFTR from K18 and CMV cassettes. CFTR protein was analyzed in 293 cells transfected with 10 μg pK18/CFTR or pCMV/CFTR. 48 hours post-transfection, CFTR was immunoprecipitated, then labeled using [^{32}P]-ATP and protein kinase A. Proteins were run on a 6% SDS polyacrylamide gel. The 293 lane depicts uninfected cells.

FIGURE 5. Inclusion of an intron 5' to the CFTR cDNA increases expression.
A hybrid intron was cloned upstream of CFTR in an expression cassette containing a viral (CMV/E1a), cell-specific (K18), or ubiquitous (UbB) promoter. 293 cells were transfected with 10 μg of each previral plasmid. 48 hours following transfection, cells were harvested for analysis. CFTR protein expression was detected by immunoprecipitation and labeling of cell extracts. Proteins were run on a 6% polyacrylamide gel. Arrow denotes region of migration of CFTR, as expected from protein standards. -, lacks hybrid intron; + contains the hybrid intron.

FIGURE 6. Analysis of CFTR expression from the K18 and CMV enhancer/promoters *in vivo*.
BALB/c mice were intranasally instilled with $2x10^9$ infectious units of Ad2/CFTR-24 or Ad2/CFTR-19 (a). A comparison of hCFTR mRNA expression from each vector was compared over the course of 45 days post-instillation by quantitative RT/PCR (b).

FIGURE 7. Ad-mediated correction of chloride current defect in CF airway epithelia. Well-differentiated airway epithelia from CF patients, cultured on air-liquid interfaces, were infected with Ad/CFTR-16, Ad/CFTR-19, or Ad/CFTR-24. CFTR expression over a course of 30 days was analyzed by correction of the defect in chloride current.

FIGURE 8. Vector diagrams of Ad2/CFTR-24αSE/wtE4 and Ad2/CFTR-24αSE/ΔE4, in which the K18 promoter/enhancer, the hybrid intron and the αSE are built into vectors which contain the wild-type E4 gene [wtE4], or are deleted for the E4 gene [ΔE4]. These constructs will have the advantages of tissue specific, high level expression, and will be viral gene product independent.

DETAILED DESCRIPTION OF THE INVENTION

Recombinant adenoviruses are currently being designed as vectors for gene transfer to a wide variety of cells and tissues, including the respiratory epithelium of patients with cystic fibrosis (CF). Successful adenoviral (Ad) gene therapy for CF will require efficient transfer of the DNA encoding the cystic fibrosis transmembrane conductance regulator (CFTR) to human airways *in vivo*. Currently, Ad-based gene transfer is limited in part by the low efficiency of entry of the viral vectors into human airway epithelia. Achieving CFTR expression levels that can impart a measurable and corrective therapeutic index may thus rely on maximizing gene expression from DNA templates that gain entry into the cell. We have tested the ability of viral vectors altered in their CFTR cassettes to provide high and sustained levels of gene expression in the lungs of immunocompetent BALB/c mice and, *in vitro,* in ciliated human airway epithelia from CF donors. We show that incorporation of a hybrid intron upstream of the CFTR cDNA and an α-globin mRNA stability element (αSE) downstream of the transcription termination site each increased the level of CFTR gene expression, both *in vitro* and *in vivo*. A keratin 18 (K18) gene enhancer/promoter and hybrid intron directed functional CFTR expression in human airway epithelia, at levels equal to that of the CMV enhancer/promoter at day 3. Unlike the CMV enhancer/promoter, whose activity declined over time, the K18 cassette provided functional and stable CFTR expression over the course of 30 days. These results suggest that correction of the CFTR defect in human airways should be attempted by using a human tissue-specific enhancer and promoter combined with *cis*-acting elements that provide more efficient mRNA stability and translatability. Enhanced expression and persistence of CFTR may be important factors in allowing lower vector doses and longer intervals between readministration.

In the present invention the K18 enhancer/promoter is used to drive transgene expression in mouse airways and, *in vitro*, in human ciliated airway epithelia. Several unexpected advantages are demonstrated in the present invention. It is demonstrated that the K18 enhancer/promoter directed transgene expression in the lung epithelial cells of BALB/c mice and provided persistent high level expression of functional CFTR in human epithelia. Additionally, CFTR expression can be markedly improved by the addition of *cis*-acting regulatory elements flanking the transgene. Further, the addition of a hybrid intron upstream of CFTR unexpectedly significantly increased gene expression from viral, cell-specific and ubiquitous promoters. An mRNA stability element from the α-globin gene 3' untranslated region also augmented CFTR expression. The 10 kb human keratin gene has been shown to be expressed in a variety of internal epithelia, including liver, lung, intestine, kidney, and the ependymal epithelium of the brain [Neznanov et al., supra]. Accordingly, The K18 enhancer/promoter, and expression cassettes containing the K18 enhancer/promoter, may also be used to target other genes to other internal epithelial tissues for other gene therapies.

Directing gene expression to a particular cell type by choosing an appropriate enhancer/promoter provides one way of targeting delivery of a therapeutic gene *in vivo*. This strategy, combined with exterior vector modifications that alter vector tropism, can increase the efficiency of adenoviral gene delivery and may allow for the ultimate use of lower vector doses in treating affected individuals. This, in turn, may diminish some subsequent adverse host effects without greatly compromising transgene expression levels. Through optimization of gene transfer and CFTR expression, one may be able to lower viral toxicity and immunogenicity and minimize the frequency of vector readministration.

It is further expected that the expression cassettes of the present invention will be useful to direct expression of other genes to the lung and airways, including the airway epithelial cells. In other embodiments of the present invention, therefore, the expression cassettes described herein are used for the targeted expression of other proteins, as desired, in the airway epithelial cells. For example, the transgene may encode one or more antimicrobial peptides ["AMPs"]. Families of AMPs which may be useful include the cecropins, defensins, and cathelins. Many AMPs which may be suitable for use in the present invention are known in the art, including: Agerberth et al., PNAS USA 92:195 (1995); Gudmundsson et al., Eur J Biochem 238:325 (1996); Gudmundsson et al., PNAS USA 92:7085 (1995); Turner et al., Antimicrob Agents Chemother 42:2206 (1998),; Cowland et al. (1995) FEBS 368:173; Gallo et al., J. Biol Chem

272:13088 (1997); Popsueva et al., FEBS 391:5 (1996); Zhao et al., FEBS 376:130 (1995); Storici et al., Biochem Biophys Res Commun 196:1363 (1993); Stukelj et al., Biol Chem Hoppe Seyler 376:507 (1995); Mahoney et al., FEBS 376:519 (1995) [cathelins]; Diamond et al., PNAS:USA (1991) 88:3952 [tracheal antimicrobial peptide]; Lehrer et al., Ann Rev Immunol 11:105 (1993) [defensins]; US Patent 5,916,872 [cyclic peptides]; US Patent 5,804,558 [protegrins]; US Patent 5,656,591; US Patent 5,459,235 [AMPs from bovine neutrophils]; US Patent 5,821,224 [AMPs from bovine neutrophils]; US Patent 5,889,152 [porphenins]; United States Patent 5,804,553 [prophenins]; US Patent 5,734,015 [family of linear AMPs from hagfish intestine]; US Patent 5,714,577 [AMPs]; US Patent 5,635,594 [gallinacins]; US Patent 5,519,115 [reverse AMPs]; US Patent 5,889,148 [antibiotic peptides]; US Patent 5,464,823 [mammalian antibiotic peptides]; US Patent 5,432,270 [DNA encoding tracheal AMPs]; US Patent 5,202,420 [tracheal AMPs]; US Patent 5,210,027; US Patent 5,032,574; US Patent 4,705,777.

The methods and compositions of the present invention may preferably include an intron, many of which are known in the art. Among those introns which may be useful as an element in the present invention are the rabbit β-globin intron [Buchman and Berg, 1988; Mol. Cell. Biol. 8:4395-4405] and one or more of the introns from the CFTR gene [Smith et al., 1996; J. Biol. Chem., 271:9947-9954]. Other introns which may be useful include hybrid introns, such as one which contains a truncated tripartite leader and splice donor site from adenovirus and a splice acceptor site from a mouse immunoglobulin gene [Yew et al., 1997; Hum. Gene Ther. 8:575-584]. Many other introns are known in the art, which may also be used in the present invention.

Other elements which may preferably be useful in the present invention include nuclear export elements, such as intronless mRNA transport elements [Huang et al., 1999; EMBO J. 18:1641-1652]; splicing enhancers, such as described in Schall and Maniatis, 1999; Mol. Cell. Biol. 19:1705-1719; and Hertel and Maniatis, Mol. Cell 1:449-455; post-transcriptional regulatory elements, such as described in Zufferey et al., 1999; J. Virol. 73:2886-2892; and 5' or 3' untranslated regions [UTR], such as described in Boado and Pardridge, 1999; Brain Res. Mol. Brain Res. 63:371-374.

The present invention includes methods for high level expression of a transgene in epithelial cells in a patient, said method comprising administering to a patient the expression constructs of the invention. Adminstration may be by any suitable means, systemic or local, including by intravenous injection, intranasal instillation, or by other acceptable means for delivery of cells, DNA, protein, or another medicament to epithelial tissue, and may vary

according to the exact nature of the disease and tissue target being treated. It is also envisioned that the constructs of the present invention may be used for *ex vivo* gene therapy, in which suitable stem cells or a patient's own epithelial cells are cultured *in vivo*, transfected with the constructs of the present invention, further cultured and then administered to a patient.

The present invention demonstrates that altering the design of the transgene cassette in adenoviral vectors can increase expression of a therapeutic gene within cells of interest, particularly epithelial cells. A tissue-specific enhancer/promoter and elements for transcriptional and translational enhancement were included in CFTR cassettes, which were investigated for the influence of those elements on gene expression in primary cultures of CF airway epithelia. The addition of the human αSE had a pronounced effect on CFTR expression from the CMV enhancer/promoter, and substitution of the CMV expression cassette with that of the K18/hybrid intron resulted in persistent and functional CFTR expression for at least 30 days in human airway epithelial cells on air/liquid interfaces [ALIs]. In *in vivo* murine experiments, CFTR expression persists even longer. Interestingly, the same elements that were effective in human cells had minimal positive or even negative effects on CFTR expression in mouse airways. In mouse models, the αSE element imparted only a slight elevation in mRNA levels, and the K18 expression cassette was much weaker than CMV. However, the vectors do persist in mouse airways *in vivo*. Although each of the human elements incorporated in the vectors contains some homology to mouse sequences [Oshima et al., supra; Wang and Liebhaber, supra], the differences appear to be relevant to enhancing gene expression in the appropriate species. These results illustrate the need for better model systems to test new putative human gene therapy vectors. Chow *et al.* have also used a K18 enhancer/promoter cassette to study transgene expression in airway cells [Chow et al., 1997, Proc. Natl. Acad. Sci. 94: 14695–14700]. Chow et al. were able to show βgal expression in the lungs of transgenic mice injected as pronuclei with a K18/βgal DNA construct. Secreted alkaline phosphatase (SEAP) and CFTR were expressed from the same promoter in transfected lung epithelial or COS cells, respectively. In the present invention, viral vectors were studied for CFTR expression and persistence in well-differentiated human airway epithelial cells. These cells were isolated directly from human CF donors and transferred to air/liquid interfaces, where they were cultured until they adopt both a transepithelial resistance and well-differentiated, ciliated morphology as determined by electron

scanning microscopy [Zabner et al., 1996, J.Virol. 70:6994-7003]. This model system more closely represents the human airway morphologically and histologically [Yamaya et al., supra].

Previous analyses of adenoviral transduction of human airway epithelia have shown that gene delivery is inefficient, most likely due to the paucity of receptors necessary for virus binding and internalization on the apical cell surface [Goldman and Wilson, 1995, J. Virol. 69:5951-5958; Pickles et al., 1998, J.Virol. 72:6014-6023; Walters et al., 1999, J. Biol. Chem. 274:10219-10226; Zabner et al., 1997, J. Clin. Invest. 100:1144–1149]. Strategies to improve adenoviral gene delivery to the lung have made use of novel mechanisms of increased vector attachment and internalization by genetic or chemical modifications to the virus [Dmitriev et al., 1998, J.Virol. 72: 9706 – 9713; Fasbender et al., 1997, J. Biol. Chem. 272:6479-6489; Kaplan et al., supra; Lee et al., 1999, Hum. Gene Ther. 10: 603 – 613; 38. Romanczuk et al., supra; Wickham et al., 1997, J. Virol. 71:8221–8229]. These types of modifications are predicted to increase the efficiency and specificity of vector delivery by targeting novel or alternate receptors. The chronic nature of a lung disease like CF necessitates another amenity in gene delivery vectors, and that is effective and persistent expression of the therapeutic gene. The effect of regulatory elements such as promoters and transcriptional and translational enhancers thus may be paramount in importance for the duration of gene expression during the lifetime of transduced well-differentiated airway epithelial cells. The frequent turnover of airway epithelial cells will necessitate vector readministration for the treatment of any chronic lung disease. Maximizing gene expression while minimizing cytotoxic vector properties will thus be important in determining the interval between vector readministration. Vectors in which all viral genes have been deleted have been shown to minimize cytotoxicity and increase persistence [Kochanek et al., 1996, PNAS USA 93:5731–5736; Schiedner et al., 1998, Nature Genetics 18:180-183]. These types of modifications, together with the changes within the transgene cassette described in the present invention, are demonstrated to greatly enhance the efficiency of therapeutic gene expression within human airways. The relative resistance of airway cells to invading foreign agents, the chronic nature of cystic fibrosis, and the relatively rapid turnover of airway epithelial cells will necessitate the implementation of several strategies for efficient therapeutic gene delivery.

The following examples are non-limiting, and are included for illustrative purposes only. The skilled artisan, having read the disclosure contained herein, will readily appreciate that many

modifications, additions and improvements are possible. Such modifications, additions and improvements are part of the present invention.

EXAMPLES

DNA and viral constructs. The K18 enhancer/promoter construct, pXKCATIs [Pankov et al., 1994; PNAS 91:873-877], was graciously provided by Dr. Robert Oshima (The Burnham Institute, La Jolla, CA). It contains the 289 base pair minimal promoter and the full-length 669 base pair enhancer from the first intron. The hybrid intron, originally obtained from pAd (Clontech), contains a truncated tripartite leader and splice donor site from adenovirus and a splice acceptor site from a mouse immunoglobulin gene [Yew et al., 1997; Hum. Gene Ther. 8:575-584]. The UbB promoter was isolated from human genomic DNA by PCR, based on the published sequence [Baker and Board, 1987; Nuc. Acids. Res. 15:443-463]. Ad2/K18/βgal was constructed by replacing the CAT gene of pXKCATIs with the βgal gene, upstream of an SV40 polyA site. The viral vector backbone contains wild-type E2 and E4 regions and a 1,549 base pair deletion of nucleotides 29292 to 30840 corresponding to the E3B gene.

The previral plasmids CMV/E1a/CFTR (+/- HI), K18/CFTR (+/- HI), and UbB/CFTR (+/- HI) each contain the first 10,670 base pairs of adenovirus type 2, with expression cassettes cloned in place of an E1 deletion (nucleotides 357 - 3328). The hybrid intron, in each case, was cloned directly upstream of the CFTR gene. CMV/E1a retains 282 base pairs of the CMV enhancer (nts -524 through -242 of the start site); the CMV promoter was replaced with the Ad2 E1a promoter, nts 351 - 545. K18/CFTR was generated by replacing the CAT gene of pXKCATIs with CFTR cDNA. UbB/CFTR was generated by cloning CFTR downstream of the UbB promoter. Each plasmid contains the SV40 polyA site downstream of CFTR. Ad2/CFTR-16 has previously been described [Scaria et al., supra]. It contains wild-type E2 and E4 genes and a 1,549 base pair deletion of nucleotides 29292 to 30840 corresponding to the E3B gene. Ad2/CFTR-19 is identical to Ad2/CFTR-16 except for the addition of the α-globin mRNA stability element (αSE) just downstream of the CFTR translation termination site. Both vectors contain a synthetic BGH polyA site. αSE is the full 3' UTR sequence, 110 nucleotides in length (Wang and Liebhaber, 1996; EMBO J. 15:5040-5051]. The αSE was isolated by PCR cloning from human placental DNA. Ad2/CFTR-24 was constructed by replacing the CMV/CFTR expression cassette with the K18/CFTR cassette; the synthetic BGH polyA site was replaced by an SV40 polyA site. The completed expression cassette within Ad2/CFTR-24 contains the 289

base pair K18 gene minimal promoter, the hybrid intron, CFTR, an SV40 polyA site, and a 669 base pair enhancer from the first intron of the K18 gene [Oshima et al., 1990; Genes & Devel. 4:835-848; Pankov et al., supra]. The viral backbone is similar to Ad2/CFTR-16 with wild-type E2 and E4 regions and a 1.6 kb deletion in the E3 region.

Cell culture. 293 cells were grown in DME high glucose with glutamine (Irvine's Scientific).

Analysis of βgal expression. For analysis of β-gal expression in airways, BALB/c mice were instilled with $1x10^9$ infectious units of Ad/K18/βgal. 3 days post-instillation, animals were sacrificed and their lungs isolated for β-gal staining and sectioning as previously described (Kaplan et al., 1998; Human Gene Ther. 9:1469 – 1479].

Analysis of CFTR expression in transduced cells. 293 cells were infected at an MOI of 5 with virus or transfected, using calcium phosphate, with 10 μg of DNA. 48 hours post-infection or transfection, cell lysates were harvested. CFTR protein was immunoprecipitated with a monoclonal antibody (mAb 24-1), then labeled with [γ^{32}P]-ATP and protein kinase A as previously described [Gregory et al., 1991; Mol. & Cell. Biol. 11:3886-3893]. Labeled CFTR protein was analyzed on SDS polyacrylamide gels. Protein levels were quantitated from the corresponding autoradiographs using a PhosphorImager (Molecular Dynamics).

CFTR mRNA analysis by RT/PCR. For comparison of CFTR mRNA expressed from Ad2/CFTR-16 and Ad2/CFTR-19 vectors, BALB/C mice were intranasally instilled with $2x10^9$ infectious units of vector in 50 μl. 3, 21, and 47 days post-instillation, animals were sacrificed and lungs were immediately frozen on dry ice and stored at -80°C. RNA was isolated and analyzed as previously described [Scaria et al., supra]. For analysis of CFTR expression from Ad2/CFTR-19 and Ad2/CFTR-24 vectors, BALB/c mice were instilled with $2x10^9$ infectious units of vector in 50 μl. 3, 21, and 45 days post-instillation, animals were sacrificed and lungs were isolated. Tissue sample collection, storage and nucleic acid extraction, and OD_{260} quantitation were performed as previously described [Scaria et al., supra]. Quantitative CFTR mRNA analysis was done using 5'nuclease TaqMan chemistry on an ABI PRISM 7700 Sequence Detection System instrument.

Reverse Transcriptase Reaction. 2 μg total RNA from each sample was treated with RNase-free DNase I (Promega) to remove any contaminating DNA. Following DNase treatment, the RNA sample was divided into two tubes; one aliquot from each sample was processed in the

absence of reverse transcriptase as a negative control. Reverse transcription was performed using the Promega Reverse Transcription Kit and 12.5 μM of a vector specific reverse primer, 1660rt (5'-TAAACATCTGCTCAAA-3') (SEQ ID NO:1), according to the manufacturer's protocol. 2.5 μls of the RT reaction were run in the amplification reaction described below. Each RT^+ sample was tested in duplicate; the "no RT" control was tested once.

Real-Time PCR amplification. Our real-time assay to measure vector CFTR expression employs the 5'-3' exonuclease activity of Taq polymerase to cleave an oligonucleotide probe labeled with fluorescent reporter and quencher molecules during the extension phase of the amplification reaction [Heid et al., 1996, Genome Research 6:986-994; Livak et al., 1995, PCR Methods and Applications 4:357-362]. The fluorescence of the reactions were measured in real-time on an ABI PRISM 7700 Sequence Detection System instrument where fluorochromes were excited using a 488nM argon laser and fluorescent emission spectra measured using a spectrograph and a CCD camera. cDNA samples were amplified in 50μl reactions containing 25 μl of TaqMan Universal PCR Master Mix (PE Biosystems) with 300 nM of each primer JMTM98-01 (5'- TGAGCAGGGAGAGGCGATA -3') (SEQ ID NO:2) and JMTM98-02 (5'- GTTCAGGACAGACTGCCTCCTT -3') (SEQ ID NO:3), 200nM of TaqMan probe 77oJM-3 (5'FAM-CCAGTGCTGATCACGCTGATGCG-TAMRA-3') (SEQ ID NO:4), 2.5U Ampli*Taq* Gold polymerase, 0.5U AmpErase UNG and 10% glycerol. Samples were run in 96-well MicroAmp optical reaction plates and caps (PE Biosystems). All reactions were kept on ice until loaded into the 7700 instrument. The reactions were held at 50 °C for 2 minutes to allow UNG activity to remove any dUTP containing contaminates in the reactions followed by a 10 minute incubation at 95°C to activate AmpliTaq Gold. These incubations were followed by 40 cycles of 95° C for 15 seconds and 60° C for 1 minute. In addition to study samples, water negative controls were run to demonstrate that the reaction components were free of contamination. Sample and standard C_T values were exported to an Excel spreadsheet (Microsoft Corp., Redmond, WA) and sample values determined from a standard curve by interpolation.

Establishing human airway epithelia. Airway epithelial cells were obtained from CF bronchi obtained after lung transplantation. Cells were isolated by enzyme digestion as previously described [Kondo et al., 1991; Am. J. Physiol. 261:L106-17; Zabner, et al., 1996, J.Virol. 70:6994-7003]. Freshly isolated cells were seeded at a density of $5x10^5$ cells/cm^2 onto collagen-coated, 0.6 cm^2 diameter millicell polycarbonate filters (Millipore Corp., Bedford, MA).

The cells were maintained at 37°C in a humidified atmosphere of 7% CO_2 and air. Twenty-four hours after plating, the mucosal media was removed and the cells were allowed to grow at the air-liquid interface [Yamaya et al., 1992; Am.J.Physiol. 262: L713 - L724]. The culture medium consisted of a 1:1 mix of DMEM/Ham's F12, 5% Ultraser G (Biosepra SA, Cedex, France), 100 U/ml penicillin, 100 µg/ml streptomycin, 1% nonessential amino acids, and 0.12 U/ml insulin. Epithelia were tested for transepithelial resistance, and for morphology by scanning electron microscopy. The genotype of the CF airway was ΔF508/ΔF508 (Genzyme Genetics).

Viral infection of human airway epithelia. Epithelia on ALIs were allowed to reach confluence and develop a transepithelial electrical resistance (Rt), indicating the development of tight junctions (Rt > 500 Ω.cm^2) and an intact barrier. 14 days after seeding, the filters containing the cells were inverted (44), and 25 MOI of Ad2/CFTR-16, Ad2/CFTR-19 or Ad2/CFTR-24 in a volume of 25 µl were added to the bottom of the Millipore filter (at this volume, the virus suspension remains on the basolateral surface). After 30 min, the cultures were rinsed thoroughly and placed back on the media right side up. Since the adenovirus diameter is only 70 to 100 nm [Shenk, 1996; Adenoviruses and Their Replication, p. 2111 - 2148. *In* B.N. Fields, D.M. Knipe, and P.M. Howley (ed.) Fields Virology, Third Edition. Lippincott - Raven Publishers, Philadelphia], it should be able to pass freely through the Millipore filters (0.4 µm pores) used to grow the airway epithelia.

Measurement of transepithelial electrical properties. Epithelia were mounted in modified Ussing chambers (Jim's Instruments, Iowa City, IA) as previously described [Zabner et al., 1998; Mol. Cell 2:397 – 403]. Epithelia were bathed on the submucosal surface with a Ringer's solution containing, in mM: 135 NaCl, 2.4 KH_2PO_4 0.6 KH_2PO_2 1.2 $CaCl_2$, 1.2 $MgCl_2$, 10 Hepes (titrated to pH 7.4 with NaOH) and 10 dextrose. The mucosal solution was identical with the exception that NaCl was replaced with 135 Na gluconate. Amiloride (10 µM) was added to the mucosal solution to inhibit Na^+ channels and thereby transepithelial Na^+ transport. The cAMP agonists, 10 µM forskolin and 100 µM IBMX, were added to the mucosal and submucosal solutions to stimulate transepithelial Cl^- current through CFTR Cl^- channels. To assess total Cl^- current, 100 µM bumetanide was then added to the submucosal solution and the change in current was measured.

RESULTS

The addition of an mRNA stability element increases CFTR expression. Adenovirus-mediated delivery of a functional CFTR gene to the airway epithelia of patients with cystic fibrosis has shown some promise, based on the evidence for subsequent expression of the therapeutic gene [reviewed in Crystal 1995; Science 270:404 – 410; Curiel et al., 1996, Am. J. Respir. Cell Mol. Biol. 14:1-18]. The results, however, suggest that the efficiency of gene transfer is poor, and that the levels of CFTR are still below the amount required for effective therapy *in vivo* [reviewed in Boucher 1999, J. Clin. Invest. 103:441 – 445]. In an attempt to optimize functional CFTR delivery via adenoviral vectors, we chose to examine molecular mechanisms for enhancing gene expression. For the studies shown here, we tested whether expression of CFTR could be improved by additions of *cis*-acting regulatory elements to the encoding gene. We began by adding a 110 base pair element from the 3' untranslated region (UTR) of the human α-globin gene downstream of CFTR in a cassette containing the strong and pan-active CMV enhancer/promoter [Schmidt et al., 1990; Mol. & Cell. Biol. 10:4406-4411. The α-globin element chosen is a cytidine-rich segment that forms an RNP complex (α-complex) correlated with a long mRNA half-life in erythroid cells [Schmidt et al., 1990; Mol. & Cell. Biol. 10:4406-4411]. The protein components of the α-complex are highly conserved from mouse to human, and similar sequences in unrelated genes of other species also confer mRNA stability [Holcik and Liebhaber, 1997; Proc. Natl. Acad. Sci. 94:2410-2414; Wang and Liebhaber, 1996; EMBO J. 15:5040-5051]. It is therefore believed that the α-complex may function similarly in a broad array of cell types and species. In order to test whether this element could enhance expression from our vectors, it was first cloned just downstream of the CFTR transcription termination site (Fig. 1). The transgene expression cassette was then incorporated into a full-length viral vector, Ad2/CFTR-19, with a wild-type E4 region and partially deleted E3B. A product of the E4 transcription unit has been implicated in the persistence of gene expression from the CMV enhancer/promoter [Armentano et al., 1997; J.Virol. 71:2408 – 2416; Brough et al., 1997; J. Virol. 71:9206-9213], a property that can be delineated to E4ORF3 [Armentano et al., 1999; J. Virol. 73:7031-7034]. E3 gene products do not appear to be necessary for this persistence in the mouse lung [Scaria et al., supra]. 293 cells were infected with Ad2/CFTR-16 or Ad2/CFTR-19 at an MOI of 5. 48 hours post-infection, cell lysates were harvested and lysed. CFTR protein was isolated by immunoprecipitation with a monoclonal antibody (mAb 24-1),

followed by labeling with [γ-^{32}P]-ATP and protein kinase A, as previously described [Gregory et al., 1991; Mol. & Cell. Biol. 11:3886-3893]. The resulting mixture was separated on an SDS polyacrylamide gel, and labeled proteins were analyzed by autoradiography. Figure 1 shows an approximate 3-fold increase in CFTR expression in these cells as a result of the addition of the α-stability element (αSE) in Ad2/CFTR-19.

To determine the effect of the αSE on levels of CFTR expression *in vivo*, 2 x 10^9 infectious units of Ad2/CFTR-19 or Ad2/CFTR-16 were instilled intranasally in BALB/c mice. Ad2/CFTR-16 has previously been shown to effectively direct long-term CFTR expression in immunocompetent mice [Scaria et al., supra]. Expression and persistence of CFTR mRNA from each vector was determined quantitatively by RT-PCR. Fig. 2 shows a slightly increased level of CFTR expression in mouse lungs as a consequence of the presence of the α-SE (CF-19 versus CF-16), a pattern that persisted throughout the course of the experiment. The overall increase in transgene expression from both vectors at a later timepoint (day 47) is a phenomenon occasionally observed in the lung with other CMV/CFTR/E4$^+$ vectors [Scaria et al., supra].

The human K18 enhancer/promoter directs gene expression in mouse airway epithelial cells. Previous studies of transgene expression have often relied on the CMV enhancer/promoter, due to its strength relative to other promoters and its activity in a variety of tissues [Schmidt et al., 1990, Mol. & Cell. Biol. 10: 4406–4411]. Persistent expression from this regulatory element can be achieved in some tissues [Armentano et al., 1999, J. Virol. 73:7031-7034; Scaria et al., supra; Schmidt et al., supra.] In many cases, however, expression often appears transient in nature, due, at least in part, to its inhibition by regulatory cytokines [Loser et al., 1998, J. Virol. 72:180–190; Qin et al., 1997, Human Gene Ther. 8: 2019–2029]. Because of this inconsistency and complexity, we replaced the CMV enhancer/promoter upstream of CFTR with the keratin 18 (K18) gene enhancer/promoter. We chose K18 due to its reported epithelial cell specificity [Broers et al., 1989, Differentiation 40:119-128; Neznanov et al., supra]. Additionally, the use of a cellular promoter is likely to be less sensitive to adverse cytokine effects, as shown for the β-actin promoter [Qin et al., supra].

To determine the strength and specificity of the K18 enhancer/promoter in airways, a recombinant viral vector was constructed in which the E1 gene product was replaced by a reporter gene for easy detection of expression. Ad2/K18/βgal contains the βgal gene driven by the K18 enhancer/promoter. BALB/c mice were instilled with 1x10^9 infectious units

Ad2/K18/βgal. 3 days post-instillation, mice were sacrificed for analysis of βgal expression in the lungs. As shown in Figs. 3a and 3b, βgal is readily expressed from this cassette in mouse airways, and the majority of the expression is localized within the epithelial cells (Fig. 3c). Based on these results, the K18 enhancer/promoter was selected as an appropriate regulatory element for directing CFTR expression in human cells.

CFTR expression from the K18 enhancer/promoter is upregulated by the addition of an upstream hybrid intron. To test for adenoviral-mediated CFTR expression using the K18 enhancer/promoter, an expression cassette for CFTR expression was ligated into a previral plasmid, pK18/CFTR, which contains the first 10,670 base pairs of the adenovirus type 2 genome with the exclusion of the transforming genes (nucleotides 357-3328). Human 293 cells were transfected with 10 μg of K18/CFTR or CMV/CFTR plasmid DNA. CMV/CFTR contains CFTR cDNA under control of the CMV enhancer/promoter, as in Ad2/CFTR-16. 48 hours after transfection, vector-mediated expression was assessed by immunoprecipitating CFTR from cellular extracts, then labeling the immunoprecipitates with [γ-^{32}P]-ATP and protein kinase A as previously described [Gregory et al., supra]. The resulting mixture was analyzed on an SDS-polyacrylamide gel for CFTR, whose fully phosphorylated form runs with an apparent molecular weight of 160 kD. Fig. 4 shows that in contrast to CMV/CFTR, CFTR expression from the K18 enhancer/promoter is barely detectable above background levels (293 cells). This contrasts with the high level of βgal expression from the same enhancer/promoter *in vitro* (not shown) and *in vivo* (Fig. 3). From these results, we questioned whether expression from CFTR cDNA may be somewhat crippled. In an attempt to increase the expression of CFTR from the K18 enhancer/promoter, an intron was inserted into the expression cassette, positioned in front of the CFTR gene. Such an effect is sometimes seen, as in the early observation that a rabbit β-globin cDNA within a recombinant SV40 virus was not expressed unless an intron was added to the transcription unit [Buchman and Berg, supra]. The CFTR genomic gene encompasses 24-exons in 250,000 base pairs of DNA [Riordan et al., 1989, Science 245:1066 –1073]. The intron-less cDNA encompasses 4.5 kilobases [Rich et al., 1993, Human Gene Ther. 4:461-476]. In the present invention, it was investigated whether a 3' mRNA stability element could provide better CFTR expression (Fig. 2). At the same time experiments were performed to determine whether the inclusion of a 5' intron would also be beneficial.

Introns within eukaryotic genes have been implicated in many functions, including protection of pre-mRNAs from degradation in the nucleus and targeting mRNAs for export to the cytoplasm. In order to determine whether these sequences are important for CFTR expression in eukaryotic cells, we added a hybrid intron to several of our cassettes. The intron we used is a 500 base pair hybrid fragment encompassing a truncated adenoviral tripartite leader sequence and splice donor site and a mouse immunoglobulin gene splice acceptor site. The inclusion of this intron was previously shown to increase CAT expression from a CMV promoter in mouse epithelial cells [Yew et al., supra]. A hybrid intron was added directly upstream of the CFTR cDNA in a vector with either a viral (CMV/E1a), cell-type specific (K18), or ubiquitous (Ubiquitin B) promoter. Each previral plasmid was then transfected into 293 cells, in parallel with a similar vector lacking the hybrid intron in the transgene cassette. CFTR expression was analyzed by immunoprecipitation and labeling of the CFTR protein from cell lysates 48 hours after transfection. Fig. 5 shows that the addition of the hybrid intron upstream of CFTR greatly enhanced gene expression from each enhancer/promoter tested.

Expression of CFTR in mouse airways is persistent but low when driven by the constitutive human K18 enhancer/promoter. In the present invention, it was shown that the human K18 enhancer/promoter directs efficient βgal expression in mouse airways (Fig. 3). It was further demonstrated that CFTR is efficiently expressed from the same promoter in human 293 cells, provided an intron is added upstream of the gene (Fig. 5). It was thus of interest to test K18-directed CFTR expression and persistence *in vivo*. In order to examine these effects, a full-length recombinant adenoviral vector, Ad2/CFTR-24, was constructed, which contains the K18 enhancer/promoter, the hybrid intron upstream of the CFTR cDNA, and a wild-type E2/E4 viral backbone (Fig. 6a). BALB/c mice were instilled intranasally with $2x10^9$ infectious units of Ad2/CFTR-24 or Ad2/CFTR-19, and CFTR mRNA persistence over the course of 45 days was measured by quantitative RT-PCR (Fig. 6b). Although both vectors encoded relatively stable levels of CFTR mRNA over the course of the experiment, the K18 cassette appeared greatly inferior to CMV/CFTR in terms of absolute amounts of encoded mRNA. Previous experiments in the present invention demonstrated the capacity for high-level CFTR expression in human cells from the same K18 cassette (Fig. 5). In order to investigate whether the discrepancy in these two systems was possibly due to a species-specific phenomenon, K18 and CMV-driven vectors were compared in human airway epithelia.

CFTR expression in human airway epithelia is enhanced by an mRNA stability element and stabilized from a human K18 promoter. Airway epithelia isolated directly from human CF donors and cultured on air liquid interfaces (ALIs) can develop a well-differentiated, ciliated morphology that closely resembles native epithelium [Yamaya et al., 1992, Am.J.Physiol. 262:L713-L724; Zabner et al., 1996, J.Virol. 70:6994-7003]. The cells are defective in Cl^- transport, a characteristic that can be partially corrected by adenoviral-mediated CFTR gene delivery [Yamaya et al., supra]. Previous experiments have shown that persistent adenovirus-mediated CFTR expression in mouse airway epithelia, *in vivo*, can be mediated by a combination of the CMV enhancer/promoter and the E4 region of the virus (Fig. 2; Armentano et al., 1999, J. Virol. 73: 7031 – 7034; Armentano et al., 1997, J.Virol. 71: 2408 – 2416; Brough et al., supra; Scaria et al., supra]. Ad2/CFTR-16 also has been shown to provide an initial high level of CFTR expression in differentiated human airway epithelia, *in vitro*, but that activity declines over a period of 21 days [Zabner, unpublished results]. In order to identify vectors that can provide increased and persistent CFTR expression in human airway epithelia, recombinant adenoviruses with modified expression cassettes were tested for their capacity to correct defective Cl^- transport over an extended period of time.

Well-differentiated, ciliated CF human airway epithelia were infected with an equal MOI of Ad2/CFTR-16, Ad2/CFTR-19, or Ad2/CFTR-24, then measured for correction of Cl^- current over the course of 30 days. Ad2/CFTR-16, which contains a CMV/CFTR expression cassette and wild-type E4 region, showed significant correction of Cl^- current at day 3, but this activity declined to near background levels by day 30 (Fig. 7). The addition of the human αSE to the CFTR expression cassette (Ad/CFTR-19) resulted in a significant increase in Cl^- channel activity by day 14, and a marked improvement over Ad2/CFTR-16 during the course of the experiment. Although the activity from each CMV vector declined after 14 days, Cl^- channel conductance at day 30 was sustained at relatively high levels by the addition of the αSE (Ad2/CFTR-19). The K18 vector, Ad2/CFTR-24, directed CFTR expression at a level equivalent to the CMV vectors, Ad2/CFTR-16 and Ad2/CFTR-19, at day 3. In contrast to the declining activity from each of the CMV vectors, Ad2/CFTR-24 provided functional and stable CFTR expression over the course of 30 days.

While the above examples exemplify the invention using adenoviral vectors for the persistent and tissue-specific expression of therapeutic genes to airway epithelial cells, it is expected that similarly targeted expression vectors can be prepared for use with other delivery

systems, such as retroviral vectors, including lentiviral vectors, adeno-associated viral vectors and non-vector delivery systems such as liposomes and naked DNA delivery. The viral or other components of such vectors are known in the art, and can be used with the presently described invention.

The disclosure of each and every publication mentioned in this specification is hereby incorporated by reference for the teachings contained therein.

WE CLAIM:

1. An adenoviral expression construct comprising, in operable linkage, the K18 promoter/enhancer element, an intron and a stability enhancing element.
2. The adenoviral expression construct of claim 1, additionally comprising a transgene.
3. The adenoviral expression construct of claim 2, wherein the transgene is the human cystic fibrosis transmembrane conductance regulator gene.
4. The adenoviral expression construct of claim 2, wherein the transgene is a gene encoding an antimicrobial peptide.
5. An adenoviral expression construct comprising, in operable linkage, the K18 promoter/enhancer element, an intron, a transgene, and a stability enhancing element.
6. The adenoviral expression construct of claim 5, wherein the transgene is the human cystic fibrosis transmembrane conductance regulator gene.
7. The adenoviral expression construct of claim 6, wherein the intron is a hybrid intron which contains a truncated tripartite leader and splice donor site from adenovirus and a splice acceptor site from a mouse immunoglobulin gene.
8. The adenoviral expression construct of claim 7, wherein the stability enhancing element is the α-globin mRNA stability element.
9. The adenoviral expression construct of claim 5, wherein the transgene is a gene encoding an antimicrobial peptide.
10. The adenoviral expression construct of claim 9, wherein the intron is a hybrid intron which contains a truncated tripartite leader and splice donor site from adenovirus and a splice acceptor site from a mouse immunoglobulin gene.
11. The adenoviral expression construct of claim 10, wherein the stability enhancing element is the α-globin mRNA stability element.
12. A method for expression of a transgene in epithelial cells in a patient, said method comprising administering to a patient the expression construct of claim 5.

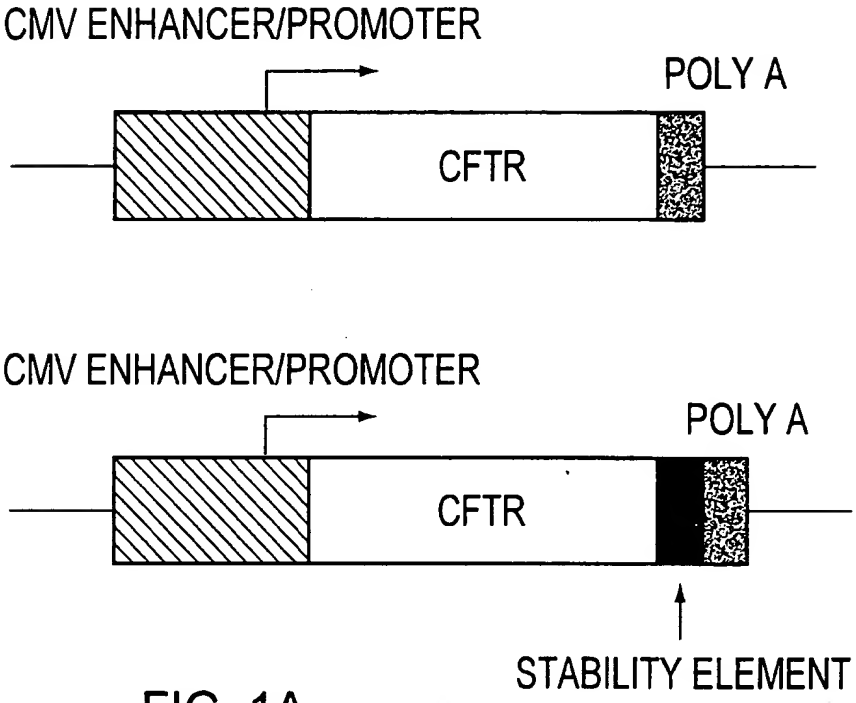
CMV ENHANCER/PROMOTER
POLY A
CFTR
CMV ENHANCER/PROMOTER
POLY A
CFTR
STABILITY ELEMENT

FIG. 1A

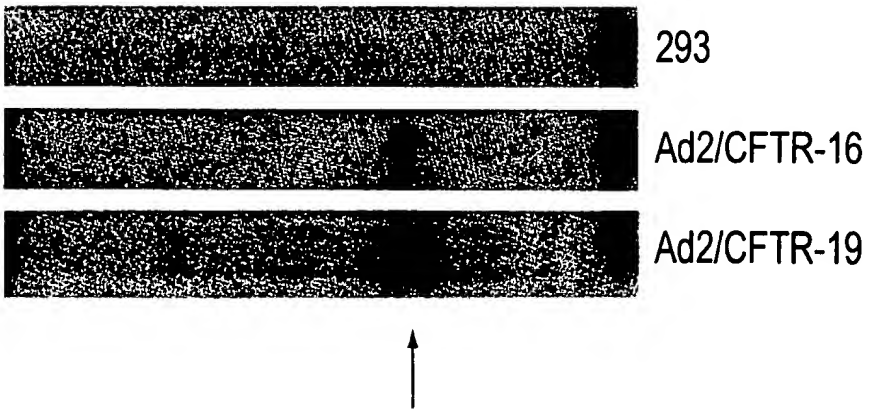
293
Ad2/CFTR-16
Ad2/CFTR-19

FIG. 1B

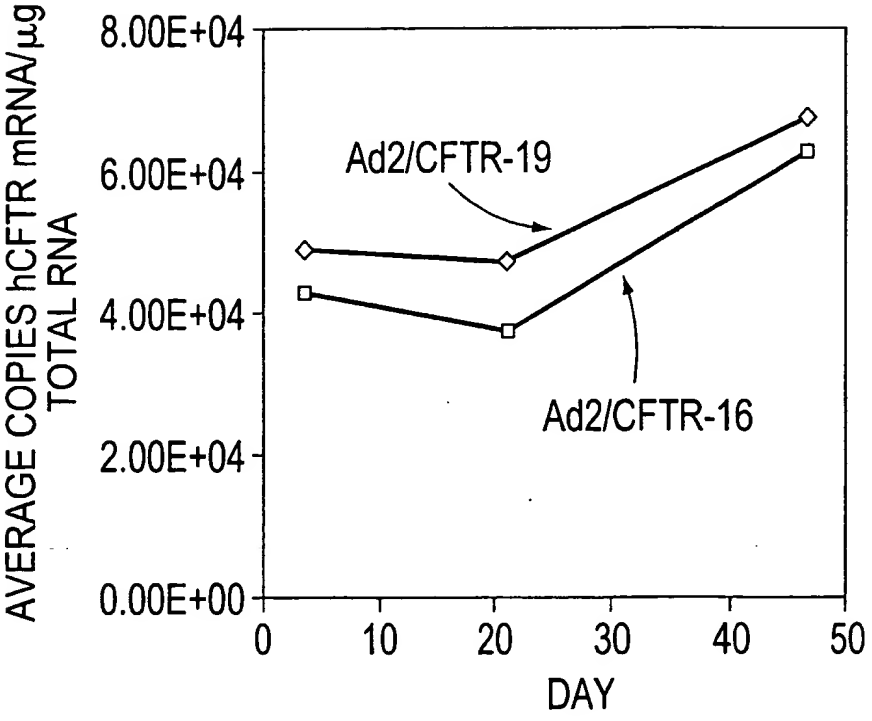
AVERAGE COPIES hCFTR mRNA/µg TOTAL RNA
8.00E+04
6.00E+04
4.00E+04
2.00E+04
0.00E+00
0
10
20
30
40
50
DAY
Ad2/CFTR-19
Ad2/CFTR-16

FIG. 2

FIG. 3C

FIG. 3A

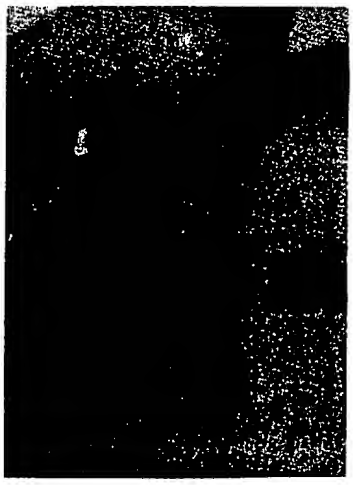

FIG. 3B

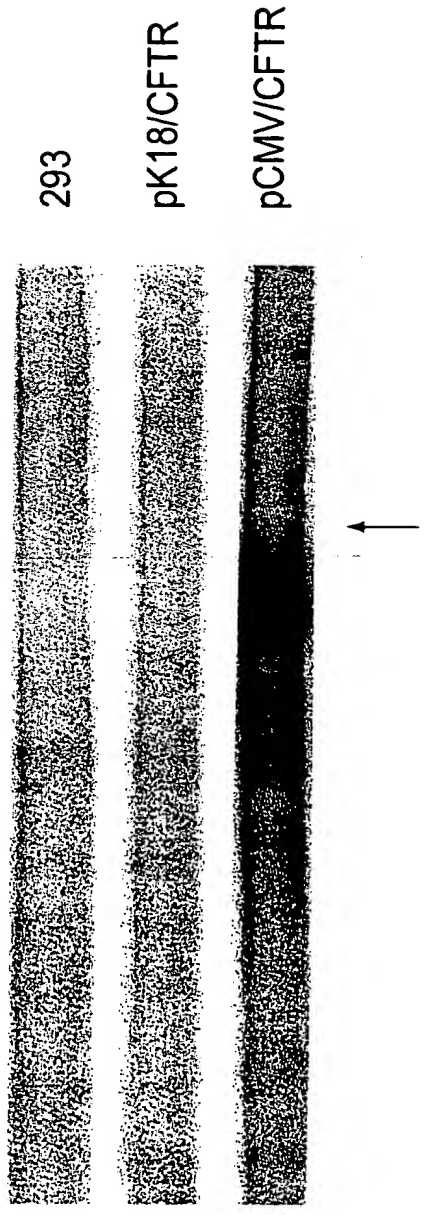

FIG. 4

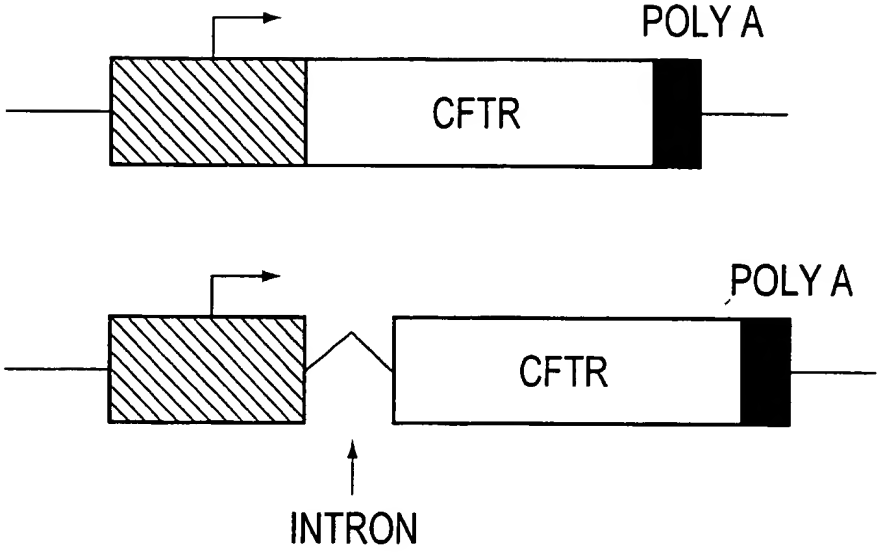

FIG. 5A

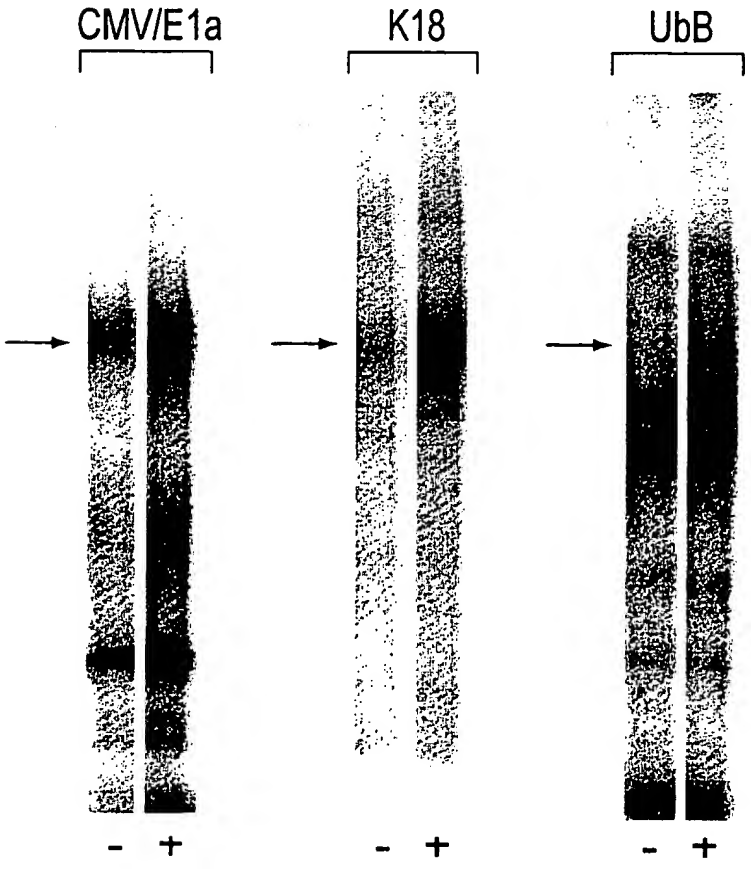

FIG. 5B

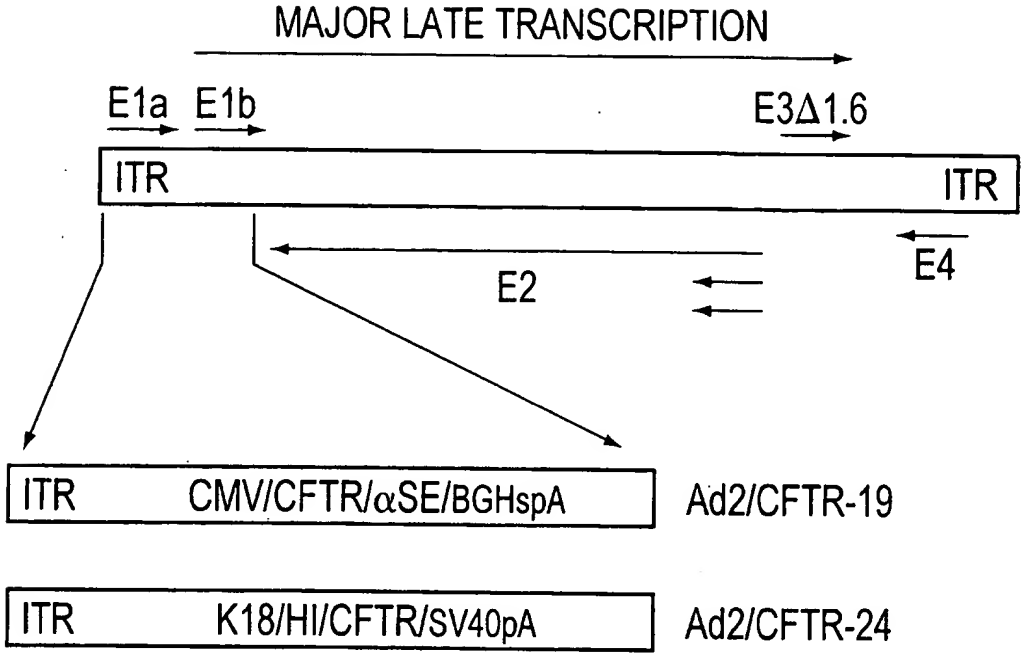
MAJOR LATE TRANSCRIPTION
E1a
E1b
E3Δ1.6
ITR
ITR
E4
E2
ITR
CMV/CFTR/αSE/BGHspA
Ad2/CFTR-19
ITR
K18/HI/CFTR/SV40pA
Ad2/CFTR-24

FIG. 6A

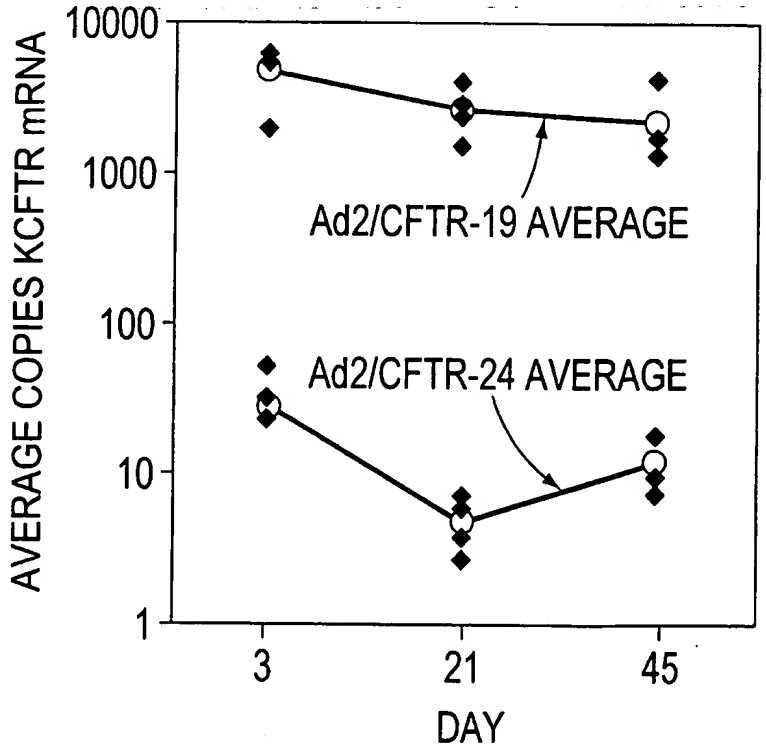
AVERAGE COPIES KCFTR mRNA
10000
1000
100
10
1
Ad2/CFTR-19 AVERAGE
Ad2/CFTR-24 AVERAGE
3
21
45
DAY

FIG. 6B

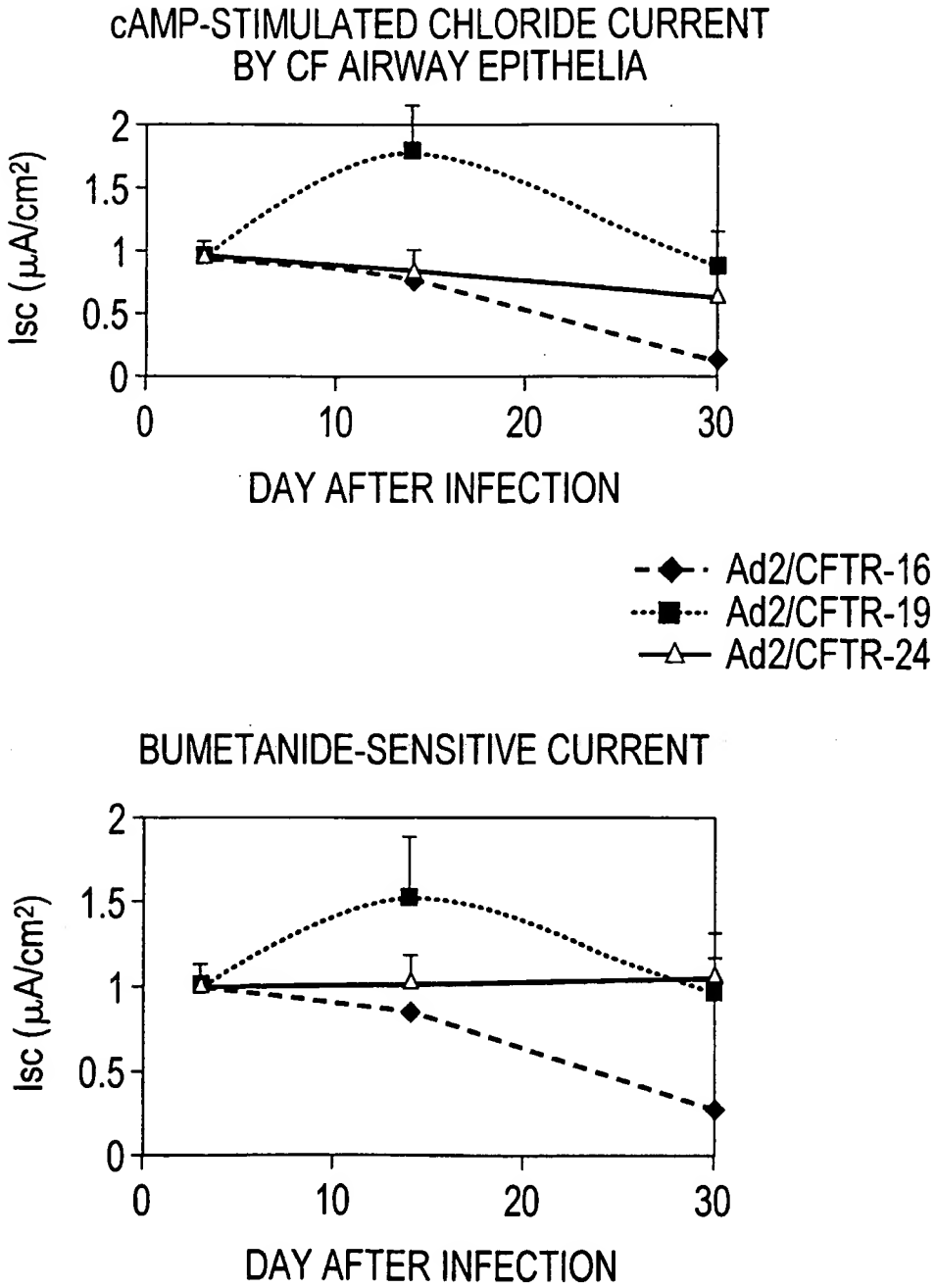
cAMP-STIMULATED CHLORIDE CURRENT
BY CF AIRWAY EPITHELIA
Isc (μA/cm²)
2
1.5
1
0.5
0
0
10
20
30
DAY AFTER INFECTION
Ad2/CFTR-16
Ad2/CFTR-19
Ad2/CFTR-24
BUMETANIDE-SENSITIVE CURRENT
Isc (μA/cm²)
2
1.5
1
0.5
0
0
10
20
30
DAY AFTER INFECTION

FIG. 7

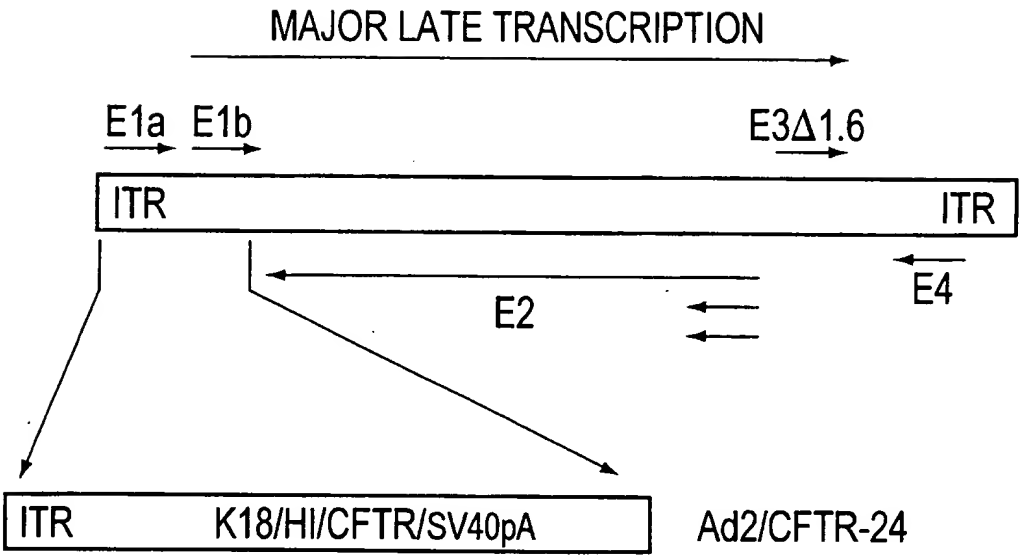
MAJOR LATE TRANSCRIPTION
E1a
E1b
E3Δ1.6
ITR
ITR
E4
E2
ITR
K18/HI/CFTR/SV40pA
Ad2/CFTR-24
Ad2/CFTR-24αSE/wtE4
Ad2/CFTR-24αSE/ΔE4

FIG. 8

SEQUENCE LISTING

<110> GENZYME CORPORATION
Romanczuk, Helen
Wadsworth, Samuel
Gregory, Richard

<120> ADENOVIRAL VECTORS MODIFIED FOR INCREASED AND PERSISTENT EXPRESSION OF THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR GENE IN HUMAN AIRWAY EPHITHELIA

<130> GA0221PCT

<150> 60/153,056
<151> 1999-08-09

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ccagtgctga tcacgctgat gcg 23

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/23692

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/861 C12N15/63 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 36545 A (RIORDAN CATHERINE R O ;ARMENTANO DONNA (US); GENZYME CORP (US); RO) 22 July 1999 (1999-07-22) the whole document, in particular page 15 lines 4-11 --- -/--	1-12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 February 2001	22/02/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Julia, P

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/23692

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHOW Y-H ET AL: "Development of an epithelium-specific expression cassette with human DNA regulatory elements for transgene expression in lung airways" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 26, no. 94, 23 December 1997 (1997-12-23), pages 14695-14700, XP002078379 ISSN: 0027-8424 cited in the application the whole document	1-12
Y	SCARIA A ET AL: "Adenovirus -mediated persistent cystic fibrosis transmembrane conductance regulator expression in mouse airway epithelium" JOURNAL OF VIROLOGY,THE AMERICAN SOCIETY FOR MICROBIOLOGY,US, vol. 72, no. 9, 1998, pages 7302-7309, XP002147592 ISSN: 0022-538X cited in the application the whole document	1-12
A	WO 97 09441 A (PIRAINO SUSAN ;VINCENT KAREN (US); GENZYME CORP (US); KYOSTIO SIRK) 13 March 1997 (1997-03-13) the whole document, in particular page 12 lines 21-24, page 15 lines 25-31 and page 18 lines 6-9	1,2,5,6, 12
A	X. WANG AND SA LIEBHABER: "Complementary change in cis determinants and trans factors in the evolution of an mRNP stability complex" EMBO JOURNAL, vol. 15, no. 18, 16 September 1996 (1996-09-16), pages 5040-5051, XP000941989 cited in the application the whole document	1,5,8,11
A	NS YEW ET AL., : "Optimization of plasmid vectors for high-level expression in lung epithelial cells" HUMAN GENE THER , vol. 8, no. 5, 20 March 1997 (1997-03-20), pages 575-584, XP000940966 cited in the application the whole document	1-3,5-7, 10,12

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/23692

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9936545 A	22-07-1999	AU 2321999 A	02-08-1999
		EP 1044274 A	18-10-2000
WO 9709441 A	13-03-1997	AU 715543 B	03-02-2000
		AU 6917396 A	27-03-1997
		CA 2230758 A	13-03-1997
		EP 0850313 A	01-07-1998
		JP 11514853 T	21-12-1999